FORM PTO-1390 (REV. 11-2000) U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371

ATTORNEY 'S DOCKET NUMBER	U.S. APPLICATION NO. (If known, see 37 CFR 1.5
118.12-US-WO	09/830691

INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/KR99/00265	29 May 1999 (29.05.99)	31 OCT 1998 (31.10.98)

TITLE OF INVENTION: VECTOR FOR THE TRANSFORMATION OF PHAFFIA RHODOZYMA AND PROCESS OF TRANSFORMATION THEREBY

APPLICANT(S) FOR DO/EO/US: Eui-Sung Choi, Sang-Ki Rhee, Jung-Hoon Sohn, Soo-Dong Park, Yoon Hyoung Lee, Seung Jae Lee, Jae-Kweon Jang, Seok Keun Choi, and Young Rok Son

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. [XX] This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. [ ] This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. [XX] This is an express request to begin national examination procedures (35 U.S.C. 371(f)). The submission must include items (5), (6), (9) and (21) indicated below.
4. [ ] The US has been elected by the expiration of 19 months from the priority date (Article 31).
5. [X] A copy of the International Application as filed (35 U.S.C. 371(c)(2))
   a. [XX] is attached hereto (required only if not communicated by the International Bureau).
   b. [ ] has been communicated by the International Bureau.
   c. [ ] is not required, as the application was filed in the United States Receiving Office (RO/US).
6. [ ] An English language translation of the International Application as filed (35 U.S.C. 371(c)(2)).
   a. [ ] is attached hereto.
   b. [ ] has been previously submitted under 35 U.S.C. 154(d)(4).
7. [XX] Amendments to the claims of the International Aplication under PCT Article 19 (35 U.S.C. 371(c)(3))
   a. [ ] are attached hereto (required only if not communicated by the International Bureau).
   b. [ ] have been communicated by the International Bureau.
   c. [ ] have not been made; however, the time limit for making such amendments has NOT expired.
   d. [X] have not been made and will not be made.
8. [ ] An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371 (c)(3)).
9. [XX] An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).
10. [ ] An English lanugage translation of the annexes of the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11 to 20 below concern document(s) or information included:

11. [ ] An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
12. [X] An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
13. [X] A FIRST preliminary amendment.
14. [ ] A SECOND or SUBSEQUENT preliminary amendment.
15. [ ] A substitute specification.
16. [ ] A change of power of attorney and/or address letter.
17. [X] A computer-readable form of the sequence listing in accordance with PCT Rule 13ter.2 and 35 U.S.C. 1.821 - 1.825.
18. [ ] A second copy of the published international application under 35 U.S.C. 154(d)(4).
19. [ ] A second copy of the English language translation of the international application under 35 U.S.C. 154(d)(4).
20. [X] Other items or information:
   (a) Computer Readable (1 diskette) and Paper Form (5-pages) of Sequence Listing;
   (b) Statement Regarding Biological Deposit – 2 pages;
   (c) One (1) KCTC Receipt for Original Deposit; and
   (d) Five (5) Sheets of Formal Drawings.

U.S. APPLICATION NO (If known, see 37 CFR 1.5) 09/830691	INTERNATIONAL APPLICATION NO PCT/KR99/00265	ATTORNEY'S DOCKET NUMBER 118.12-US-WO

21. XX The following fees are submitted:	CALCULATIONS PTO USE ONLY	
BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)):		
Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO **$1000.00**		
International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO **$860.00**		
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International preliminary examination fee (37 CFR 1.482) paid to USPTO but all claims did not satisfy provisions of PCT Article 33(1)-(4) **$690.00**		
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ENTER APPROPRIATE BASIC FEE AMOUNT =	$ 1000.00	
Surcharge of **$130.00** for furnishing the oath or declaration later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492(e)).	$	

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE	$	
Total claims	13 - 20 =		x **$18.00**	$	
Independent claims	3 - 3 =		x **$80.00**	$	
MULTIPLE DEPENDENT CLAIM(S) (if applicable)			+ **$270.00**	$	
TOTAL OF ABOVE CALCULATIONS =				$ 1000.00	
☐ Applicant claims small entity status. See 37 CFR 1.27. The fees indicated above are reduced by 1/2. +				$	
SUBTOTAL =				$	
Processing fee of **$130.00** for furnishing the English translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492(f)).				$	
TOTAL NATIONAL FEE =				$ 1000.00	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). **$40.00** per property +				$ 40.00	
TOTAL FEES ENCLOSED =				$ 1040.00	
				Amount to be refunded:	$
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a. ☐ A check in the amount of $ _____ to cover the above fees is enclosed.

b. ☐ Please charge my Deposit Account No. _____ in the amount of $ _____ to cover the above fees. A duplicate copy of this sheet is enclosed.

c. XX The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 50-0494 . A duplicate copy of this sheet is enclosed.

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NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137 (a) or (b)) must be filed and granted to restore the application to pending status.

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SIGNATURE

Karen S. Canady
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39,927
REGISTRATION NUMBER

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant:	Eui-Sung Choi et al.	Examiner:	To be assigned
Serial No.:	To be assigned	Group Art Unit:	To be assigned
Filed:	To be assigned	Docket:	G&C 118.12-US-WO
Title:	VECTOR FOR THE TRANSFORMATION OF PHAFFIA RHODOZYMA AND PROCESS OF TRANSFORMATION THEREBY		

CERTIFICATE OF MAILING UNDER 37 CFR 1.10
'Express Mail' mailing label number: EL816010360US
Date of Deposit: April 26, 2001
I hereby certify that this paper or fee is being deposited with the United States Postal Service 'Express Mail Post Office To Addressee' service under 37 CFR 1.10 and is addressed to the Commissioner for Patents, Washington, D.C. 20231.
By: Darlene Ross
Name: Darlene Ross

PRELIMINARY AMENDMENT

BOX PCT
Commissioner for Patents
Washington, D.C. 20231

Dear Sir:

Prior to a first Office Action, please amend the above-identified application as follows:

IN THE CLAIMS

Please amend claims 4, 7, 8, 11 and 12 as follows:

1. (UNCHANGED) An L41 gene encoding a *Phaffia rhodozyma* ribosomal protein whose amino acid sequence is described by SEQ ID NO: 3.

2. (UNCHANGED) The L41 gene of claim 1, wherein the genomic sequence of the gene is described by SEQ ID NO: 1.

3. (UNCHANGED) The L41 gene of claim 1, wherein the cDNA sequence of the gene is described by SEQ ID NO: 2.

4. (AMENDED) The L41 gene of claim 1, wherein the codon[s] representing the amino acid sequence at position 56 is replaced by [the codons] a codon representing glutamine.

5. (UNCHANGED) A ribosomal DNA of *Phaffia rhodozyma*, which is described by SEQ ID NO: 4.

6. (UNCHANGED) A vector for transforming *Phaffia rhodozyma*, comprising a cycloheximide-resistant gene and a portion of *Phaffia rhodozyma* ribosomal DNA.

7. (AMENDED) The vector of claim 6, wherein the cycloheximide-resistant gene is [the] an L41 gene [of claim 4] encoding a *Phaffia rhodozyma* ribosomal protein whose amino acid sequence is described by SEQ ID NO: 3, wherein the codon representing the amino acid sequence at position 56 is replaced by a codon representing glutamine.

8. (AMENDED) The vector of claim 6, wherein the *Phaffia rhodozyma* ribosomal DNA is [the ribosomal DNA of claim 5] described by SEQ ID NO: 4.

9. (UNCHANGED) The vector of claim 6, wherein the vector is pTPLR1 represented by figure 3.

10. (UNCHANGED) A process of transforming yeast with the vector of claim 6.

11. (AMENDED) The process of claim 10, wherein the yeast is *Phaffia rhodozyma*.

12. (AMENDED) The process of claim 10, wherein the vector [of claim 6] is cleaved into a linear form.

13. (UNCHANGED) The process of claim 10, wherein the transformation is performed by electroporation under an electric pulse of 0.8~1.2 kV, an internal resistance of 400~800 Ω, and a capacitance of 25~50 μF.

REMARKS

Prior to a first Office Action in this application, Applicants request that original claims 4, 7, 8, 11 and 12 be amended. These amendments merely remove reference to more than one previous claim and correct grammatical errors. The amendments do not involve any new matter or objectionable changes. Entry of these amendments is respectfully requested.

It is submitted that this application is now in good order for allowance and such allowance is respectfully solicited. Should the Examiner believe minor matters still remain that can be resolved in a telephone interview, the Examiner is urged to call Applicants' undersigned attorney.

Respectfully submitted,

GATES & COOPER LLP
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KSC/dr
G&C 118.12-US-WO

VECTOR FOR THE TRANSFORMATION OF *Phaffia rhodozyma* AND PROCESS OF TRASFORMATION THEREBY

FIELD OF THE INVENTION

The present invention relates to novel vectors for the transformation of *Phaffia rhodozyma* and to a process of transformation thereby. Particularly, this invention relates to an L41 gene encoding a ribosomal protein derived from *Phaffia rhodozyma* which is useful for producing natural pigment astaxanthin; an L41 gene mutated to a cycloheximide-resistant form; a *Phaffia rhodozyma* ribosomal DNA; a vector for the stable transformation of *Phaffia rhodozyma*, comprising said mutated L41 gene and said ribosomal DNA; and a process of transformation thereby.

BACKGROUND

Phaffia rhodozyma is reddish yeast species producing astaxanthin, the useful natural pigment. Astaxanthin is a member of the carotenoids, which are represented by β-carotene, the precursor of vitamin A. Naturally, astaxanthin is widely distributed, especially to Crustacea, trout and salmon as their main pigment, although they cannot synthesize astaxanthin and should be supplied with it from the diet. Thus, it has been considered necessary to add the pigment in the

cultivation of Crustacea, trout and salmon, so that the added pigments to the Crustacea and fishes may attract the consumers and give better flavors. This carotenoid pigment plays key roles in the physiological metabolism of human as well as animals, with known effects such as the precursor of vitamin A, the enhancement of immunological function, the antioxidant activity, the prevention of cancer and senescence, etc.

Because of increasing interests in *Phaffia rhodozyma* and pigments produced thereby, there have been a number of reports concerned about the culture of *Phaffia rhodozyma*. However, these researches have been focused on how the inexpensive materials can be used for its culture, and have resulted in the development of culturing methods, in which various local products may be employed, such as alfalfa juice (Okagbue *et al.*, *Appl. Microbiol. Biotechnol.*, 20, 33, 1984), molasses (Haard *et al.*, *Biotechnol. Lett.*, 10, 609, 1988), the byproducts of grape juice processing (Lango *et al.*, *Biotech. Forum Europe*, 9, 565, 1992), peat hydrolyzate (Martin *et al.*, 58, 223, 1993), the byproducts of corn wet-milling (Hayman *et al.*, *J. Ind. Microbiol.*, 14, 389, 1995), and the mixture of sugar cane extract, urea and phosphoric acid (Fontana, *et al.*, *Appl. Biochem. Biotechnol.*, 57/58, 413, 1996).

Although little is known about the genetics of *Phaffia rhodozyma*, the physiological features of

Phaffia rhodozyma have been disclosed and the *Phaffia rhodozyma* mutant has recently been selected to produce higher level of the pigment (Johnson *et al.*, *Crit. Rev. Biotechnol.*, 11, 297, 1991; An *et al.*, *Appl. Environ. Microbiol.*, 55, 116, 1989; Chumpolkulwong *et al.*, *J. Ferment. Bioeng.*, 75, 375, 1997; Lewis *et al.*, *Appl. Environ. Microbiol.*, 56, 2944, 1990). In addition, a genetic analysis enlightened the ploidy and sexual cycle of *Phaffia rhodozyma*. In a flow cytometry study, Calo-Mata and Johnson found that no strain was haploid and that most were polyploid (Calo-Mata *et al.*, *Yeast Gen. Mol. Biol. Meet.*, 126, 1996). A pedogamic sexual process of conjugation has been also described (Golubev *et al.*, *Yeast*, 11, 101, 1995).

Although *Phaffia rhodozyma* is potentially useful for the production of astaxanthin and the like, the pigment level in the wild type of *Phaffia rhodozyma* is very low. Therefore, there have been increasing attempts to develop novel mutant strain of *Phaffia rhodozyma*, which can produce the higher level of the pigment. However, these attempts have been hampered by the reduced growth rate and genetic instability, which may occur when the pigment content in a mutant exceeds over the optimal range.

Another obstacle to the progress of the mutant is the method of mutagenesis. Chemical mutagenesis

procedure has been performed conventionally, but it is associated with the simultaneous mutation of undesired genes leading to pleiotropic effects such as the reduction of growth rate, the prolongation of induction time in the fermentation, etc. Furthermore, the genome of the mutant strain is not stable, since its subculture often decreases the yield of the pigment.

To solve these problems in the conventional breeding procedures and to enlarge the applicability of *Phaffia rhodozyma*, molecular breeding approaches have been initiated recently, using genetic transformation. However, since most of *Phaffia rhodozyma* strains are polyploid and thus cannot be made to be an auxotrophic variant by the method conventionally applied to yeast, preferable is the approach employing antibiotics-resistant genes as selectable marker. More recently, there was reported a transformation system in which *Phaffia rhodozyma* actin promoter and G418-resistant gene were used for the transformation of *Phaffia rhodozyma*, although it showed poor transformation efficiency (Wery et al., Gene, 184, 89, 1997).

On the other hand, cycloheximide, an eukaryote-specific antibiotics, is applicable to the selection of yeast transformants. The target molecule of cycloheximide action is aminoacyl-tRNA binding site (A site), where it blocks peptidyl transferase activity.

As a result, it inhibits protein synthesis and cell growth in eukaryotes, without an effect on the organelles such as chloroplasts and mitochondria. Furthermore, it has been found that cycloheximide interacts with ribosomal protein L41, and that a mutation in L41 gene confers cycloheximide-resistance on the yeast transformants. Thus, cycloheximide and related mutant form of L41 gene are widely applicable to the process of transformation for yeasts.

Recent studies support the applicability of L41 gene to selectable marker in yeasts. Takagi et al. found that amino acid substitution through the mutagenesis of *Saccharomyces cerevisiae* L41 gene conferred cycloheximide-resistance, suggesting the usefulness of L41 gene as a selectable marker (Takagi *et al.*, *J. Bacteriol.*, 174, 254-262, 1992). Mutoh et al. proposed a biotechnological tool using *Candida maltosa* L41 gene as a selectable marker (Mutoh et al., *J. Bacteriol.*, 5383, 177, 1995). As it is well known that cycloheximide-resistance is conferred on *Candida utilis* as well as *Phaffia rhodozyma* by the substitution of 56th amino acid residue in the L41 protein (Keiji Kondo *et al.*, *J. Bacteriol.*, 7171, 177, 1995), transformation system thereby has been developed. Similar approaches have been attempted in *Kluyveromyces lactis* and *Schwanniomyces occidentalis* (Dehoux *et al.*, *Eur. J. Biochem.*, 213, 841-843, 1993; Pozo *et al.*, *Eur.*

J. Biochem., 213, 849-857, 1993). On algae *Tetrahymena*, the resistance is conferred by substitution of 40th amino acid residue, methionine to glutamine (Roberts *et al.*, *Exp. Cell. Res.*, 312, 81, 1973).

To overcome the foregoing and other disadvantages, we, the inventors of the present invention, have noted that cycloheximide and related mutation in L41 gene may be used to develop an efficient transformation system, in which a foreign gene is stably integrated into the genome of *Phaffia rhodozyma*, and in which the transformants are undoubtedly selected. To develop such system, we have constructed transforming vectors comprising the antibiotics-resistant gene and the targeting gene, which is used for the stable integration of foreign gene. We transformed *Phaffia rhodozyma* with such vectors, according to a modified method for electrotransforming *Cryptococcus neoformans*, a member of Basidiomycetes, of which *Phaffia rhodozyma* is also another member (Kim *et al.*, *Appl. Environ. Microbiol.*, 64, 1947, 1998).

The present invention is performed by cloning and sequencing *Phaffia rhodozyma* L41 gene; modifying the L41 gene by the mutagenesis of the region responsible to cycloheximide-resistance; constructing the vectors for transforming by inserting ribosomal DNA into the mutated L41 gene; transforming *Phaffia rhodozyma* with

the vector by electroporation method; and verifying the stable integration of the vector into the genome of the transformants.

SUMMARY OF THE INVENTION

It is an object of this invention to provide a vector for transforming *Phaffia rhodozyma* efficiently.

It is a further object of this invention to provide an antibiotics-resistant vector for transforming *Phaffia rhodozyma*, which comprises the L41 protein of *Phaffia rhodozyma*.

It is an additional object of this invention to provide a L41 gene encoding the L41 protein of *Phaffia rhodozyma*.

It is another object of this invention to provide a mutated L41 gene that can be used as a cycloheximide-resistant gene.

It is still another object of this invention to provide a ribosomal DNA of *Phaffia rhodozyma*, which can be used to enhance the integration efficiency of foreign DNA into *Phaffia rhodozyma* genomes.

It is also an object of this invention to provide a process of transforming *Phaffia rhodozyma* by electroporation.

Further objects and advantages of the present invention will appear hereinafter.

In accordance with the present invention, the foregoing objects and advantages are readily obtained.

The present invention provides an L41 gene encoding a ribosomal protein originated from *Phaffia rhodozyma*.

In addition, this invention provides a mutated L41 gene in which the amino acid at the position 56 is replaced by glutamine. Since the amino acid residue is responsible for the cycloheximide-resistance, this mutated gene in a vector is useful for a selectable marker.

This invention also provides a ribosomal DNA derived from *Phaffia rhodozyma*.

In addition, this invention provides a vector comprising a cycloheximide-resistant gene and a ribosomal DNA derived from *Phaffia rhodozyma*.

In such aspect of this invention, also provided is a vector, pTPLR1 comprising the mutated L41 gene of *Phaffia rhodozyma* and a portion of the *Phaffia rhodozyma* ribosomal DNA.

This invention also provides a process of transforming *Phaffia rhodozyma* with the vector by electroporation.

In such aspect of this invention, the vector is preferably cleaved into a linear form, and the preferable condition for electroporation is such that

electric pulse is 0.8~1.2 kV, an internal resistance is 400~800 Ω, and a capacitance is 25~50 μF.

Further features of the present invention will appear hereinafter.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is nucleotide and deduced amino acid sequences of L41 gene encoding *Phaffia rhodozyma* ribosomal protein, where

Open boxes: TATA and CAAT sequences;

Underlined: the position of primers;

Bold letters: consensus sequence in splicing region of intron;

Open circle: amino acid residue at position 56

Figure 2 represents the construction of pTPLR1 vector and its restriction map, where

Numbers in parentheses: the sizes of inserts;

Blank boxes: DNA fragment containing L41 gene;

Grey boxes: rDNA fragments;

Black boxes: exons of L41 gene;

Thin lines: pBluescript SK(+) sequence;

Horizontal arrow: transcriptional direction of L41 gene;

X: *Xba*I site; S: *Sal*I site; C: *Cla*I site;

H: *Hind*III site; E: *Eco*RI site; Xh: *Xho*I site;

Sm: *Sma*I site; Bg: *Bgl*I site; Ba: *Bal*I site;

Kp: *Kpn*I site;

Figure 3 represents the restriction map of pTPLR1, the vector of this invention,

Figure 4 represents the relationship between the condition of electroporation and the transformation efficiency or cell viability;

Figure 5 represents Southern blot analysis of pTPLR1 transformants, where

C: nontransformant control;

1 to 5: pTPLR1 transformants;

Figure 6 represents schematically the mode of pTPLR1 integrated into the chromosome.

DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS

The present invention is based upon the notion that cycloheximide and related mutation in L41 gene may be used to develop a transformation system, in which foreign gene is stably integrated into the genome of *Phaffia rhodozyma*, and in which the transformants are undoubtedly selected.

Hereinafter, the present invention is described in detail.

In one aspect, the present invention provides a L41 gene encoding a *Phaffia* ribosomal protein.

In a preferred embodiment, we have obtained genomic and cDNA sequences containing the L41 gene encoding a *Phaffia rhodozyma* ribosomal protein, and these sequences are prepared from a *Phaffia rhodozyma* strain (ATTC 24230).

The L41 gene identified in this invention shows high homology with other known L41 gene of yeasts, but contains 6 introns which have specific sequences in 5′ and 3′ regions of each intron. The genomic sequence described by SEQ ID NO: 1 contains the L41 gene of 1,223 bp, which in turn contains the cDNA sequence described by SEQ ID NO: 2. Of the deduced amino acid sequence described by SEQ ID NO: 3, proline at position 56 is responsible for the sensitivity to cycloheximide (see FIG 1).

In another preferred embodiment, the cloned L41 gene is modified by site-directed mutagenesis, so that the mutated L41 gene is made to be a cycloheximide-resistant gene, or gene which can confer resistance to cycloheximide on an acceptor organism. Particularly, the mutagenesis is performed to replace the proline residue by glutamine, at the position 56 (see FIG 2).

The mutagenesis in this invention includes all the

possible modification of triplet codon in the amino acid position 56. For example, the codons for proline 56 may be replaced by all possible triplet codons for glutamine.

This invention also provides a ribosomal DNA (hereinafter "rDNA") derived from *Paffia* yeast.

In this invention, rDNA means not only a DNA sequence which is transcribed to bear all types of eukaryotic ribosomal RNA, but also a non-transcription spacer (hereinafter, "NTS"), or a DNA sequence between the transcribed rDNA. rDNA can be preferably used to enhance the integration efficiency of foreign DNA into host genomes because rDNA sequence is highly repeated as tandem units in the eukaryotic genomes.

In a preferred embodiment, we identified the rDNA which is described by SEQ ID NO: 4. This rDNA sequence contains NTS.

This invention provides a transforming vector comprising a cycloheximide-resistant gene and a rDNA.

According to one preferred embodiment, the rDNA may be used to enhance the integration efficiency of foreign DNA into the host genome.

According to another preferred embodiment, the *Phaffia rhodozyma* L41 gene modified to cycloheximide-resistant gene is employed as a selectable marker in

the transforming vector (see fIG 2). This transforming vector is useful for the stable introduction of a foreign gene into a host genome.

More particularly, this invention provides pTPLR1, a vector for transforming yeasts, most preferably for transforming *Phaffia rhodozyma*, which comprises an NTS portion of *Phaffia rhodozyma* rDNA and a mutated *Phaffia rhodozyma* L41 gene where the codon for proline at amino acid position 56 is replaced by the codon for glutamine (see FIG 3).

The transforming vectors of this invention may be readily modified and improved within the spirits and scope of this invention. For example, the transforming vector of this invention may include diverse L41 genes modified through various mutagenesis procedures and diverse rDNA sequences originated from various organisms.

In another aspect of this invention, also provided is a process of transforming yeasts with foreign DNA. The process is based upon the established method for transforming *Cryptococcus neoformans*, but optimized to yeasts, using an antibiotics-resistance gene derived from yeasts instead of the bacterium-derived counterpart.

In a preferred embodiment, the transforming vector is cleaved into a linear form before transformation.

The restriction enzymes used and the reaction may be selected carefully so that foreign DNA is efficiently introduced into host genome and only desired sequences of the vector are inserted to the host genome.

In the transforming process of this invention, an electroporation procedure is employed. According to another embodiment, the preferable condition for electroporation is such that electric pulse is 0.8~1.2 kV, an internal resistance is 400~800 Ω, and a capacitance is 25~50 μF. After electroporation, the yeast cells are cultured at 23°C for 14~16 hours, then spread on solid medium containing cycloheximide, and further cultured at 23°C for 4~5 days. Assessing the effects of various conditions for the electroporation on the cell viability and the transforming efficiency (see FIG 4) reveals that abundant transformants are produced under such condition as electric pulse of 0.8 kV, an internal resistance of 600 Ω, and a capacitance of 50 μF.

In still another embodiment, Southern blot analysis is used to verify the stable integration of foreign DNA (see FIG 5 and 6). The result confirms that the introduced genes are stably maintained in host genome, even after multiple subcultures on the medium without cycloheximide.

EXAMPLES

Practical and presently preferred embodiments of the present invention are illustrative as shown in the following Examples.

However, it will be appreciated that those skilled in the art, on consideration of this disclosure, may make modifications and improvements within the spirit and scope of the present invention.

Example 1: The isolation of *Phaffia rhodozyma* L41 gene

To isolate genomic DNA sequence encoding *Phaffia rhodozyma* ribosomal protein L41, we synthesized two PCR (; polymerase chain reaction) primers, the sequences of which were deduced from the nucleotide sequence of other yeast L 41 genes and described by SEQ ID NO: 5 (CYH1) and SEQ ID NO: 6 (CYH3). PCR was performed in which the synthetic oligonucleotides, CYH1 and CYH3 were used as PCR primers and in which genomic DNA isolated from *Phaffia rhodozyma* (ATCC 24230) was employed as template. The PCR produced 700 bp DNA fragments containing L41 gene, which were then brought to the labeling reaction using digoxigenin (DIG)-labeling kit (Boehringer Mannheim, Germany) so as to be used as a probe for Southern blot analysis. To clone full-length L41 gene, Southern hybridization was performed as described in the work of Sambrook et al.

(Sambrook et al., Molecular Cloning, 2nd Edition, Cold Spring Harbor Laboratory Press, 1989) in a solution containing 5X SSC, 0.1% (w/v) sarcosyl, 0.02% (w/v) SDS, 5% blocking agent, and 50% (v/v) formamide, at 42°C. A strong hybridization signal was observed from an 8-kb *Xba*I fragment, and the *Xba*I fragments of 7 to 9-kb were isolated and ligated into pBluescript SK(+) (Stratagene, USA) to make a minilibrary. A clone (pTPL2), hybridizing with the PCR product was identified in a further Southern blot analysis in which the DNA fragments of the minilibrary were blotted onto the membrane.

To identify the L41 gene without intron, *Phaffia rhodozyma* L41 cDNA was isolated by the method of rapid amplification of cDNA ends (; RACE) with 3′-RACE (GIBCO BRL, USA) and 5′-RACE (Clontech, USA) kits. Total RNA was prepared by the method of Elion and Warner (Elion *et al., Cell*, 39, 663-673, 1984). Then mRNA was selected from the total RNA, using mRNA isolation kit (Novagen), and brought to 3′ RACE reaction in which synthetic oligonucleotide described by SEQ ID NO: 7 was used as 3′ RACE primer, and 5′ RACE reaction by SEQ ID NO: 8 as 5′ RACE primer.

The sequencing of the 3′ and 5′ RACE products suggested that a putative open reading frame of 1,223 bp be interrupted by six introns. The cloned L41 gene was found to show high homology with those of other

yeasts. However, the number of introns and their organization in the *Phaffia rhodozyma* L41 gene were quite different from the other yeast L41 genes, where there is only one intron. GTPuNGT sequence and PyAG sequence were conserved in 5′ and 3′ ends, respectively, of *Phaffia rhodozyma* L41 gene; this conserved sequences have also reported in the *Phaffia rhodozyma* actin introns. The *Phaffia rhodozyma* L41 gene encodes ribosomal protein comprising 106 amino acids, and most notably, proline at position 56 is identified to the amino acid residue responsible for the sensitivity to cycloheximide. The genomic DNA sequence of *Phaffia rhodozyma* L41 gene was registered in GenBank on May 19, 1997, with accession NO. AF 004672 (see FIG 1).

Example 2: Cycloheximide-resistant L41 gene

To confer the cycloheximide-resistance on L41 gene, we performed the site-directed mutagenesis which resulted in the amino acid converting proline 56 to glutamine. Specifically, mutagenesis was carried out with the QuickChange in vitro mutagenesis kit (Stratagene) as described in the manufacturer's instructions with complementary mutagenic primers corresponding to amino acids 52 to 59 and described by SEQ ID NO: 9 and 10. Digested from the 8.0-kb fragment in Example 1, the 2.2-kb *SalI* fragment was replaced

with the mutated fragment.

Example 3: The isolation of ribosomal DNA

Ribosomal DNA (rDNA) in this invention was exploited to enhance the integration efficiency of foreign DNA into *Phaffia rhodozyma* genomes. To clone the rDNA fragment, two pairs of PCR primers, described by SEQ ID NO: 11, 12 (corresponding to 18S rDNA part) and 13, 14 (corresponding to 28S rDNA part), were designed from the known partial rDNA sequence of *Phaffia rhodozyma*.

By PCR with these two pairs of primers, two DNA fragments were obtained, one of which was 1.5-kb fragment containing the 5.8S rDNA NTS (; non-transcription spacer) region with the primers described by SEQ ID NO: 11 and 14, and the other of which was 6-kb fragment containing the 5S rDNA NTS region with the primers described by SEQ ID NO: 12 and 13.

Two DNA fragments were used as a probe for cloning the rDNA unit in genomic Southern blot analysis, followed by the construction of minilibrary, as described in Example 1. Multiple rounds of Southern hybridization identified an 8.5-kb *Hin*dIII fragment, which was cloned and whose identity was confirmed by partial sequencing. A 730-bp *Xho*I and *Xba*I fragment of the 8.5-kb fragment, which spans NTS region between 5S

and 18S rDNA, was subcloned in pBluescript and the resulting vector was designated as pTPR4. The sequencing of pTPR4 enlightened that the cloned rDNA fragment showed much high homology with 5.8S and 25S rDNA region of *Candida neoformans*, a member of Basidiomycetous yeasts including *Phaffia rhodozyma*. The 730-bp nucleotide sequence of *Phaffia rhodozyma* rDNA gene was registered in GenBank on July 28, 1997, with accession NO. AF 016256.

Example 4: The construction of vector for transforming *Phaffia rhodozyma*

To construct vectors for transforming *Phaffia rhodozyma* efficiently, we exploited pTPL5 vector containing the mutated L41 gene of Example 2 and pTPR4 vector containing the rDNA fragment of Example 3 (see FIG 2). Particularly, we constructed pTPLR1 vector for transforming *Phaffia rhodozyma*, using the 3.7-kb fragment of pTPL5 as a cycloheximide-resistant marker and the 730-bp rDNA fragment of pTPR4 as a targeting sequence into *Phaffia rhodozyma* genome with multicopy. The 3.7-kb *Xba*I-*Sal*I fragment of pTPL5 containing the mutated L41 gene was treated with the Klenow enzyme and inserted into the *Bal*I site of pTPR4. The resulting plasmid, pTPLR1 (see FIG 3), was introduced into *E.*

coli DH5α strain, and the transformed *E. coli* strain was deposited in Korean Collection for Type Cultures (KCTC) on October 21, 1998 (accession NO: KCTC 0535BP).

We also constructed a plasmid, pTPLR2, which has the reverse direction of expressed sequence. The pTPLR1 and pTPLR2 vectors were digested with *Sma*I or *Bgl*I-*Kpn*I restriction enzymes, before the vector was brought to the transformation and integrated into the rDNA region of *Phaffia rhodozyma* genome.

Example 5: The transformation of *Phaffia rhodozyma* with pTPLR1 vector

To transform *Phaffia rhodozyma* with the pTPLR1 vector efficiently, we developed the transformation method, which is based upon the method for transforming a Basidiomycetous yeast, *Cryptococcus neoformans* (Varma *et al., Infect. Immun.*, 60, 1101, 1992) but optimized for *Phaffia rhodozyma*. Electroporation procedure was employed in the process of this invention. Particularly, *Phaffia rhodozyma* cells from a log-phase cluture in 50 ml of YM medium were harvested by centrifuge at 3,000 rpm for 10 minutes, then washed twice with equal volume of electroporation buffer (270 mM sucrose, 10 mM Tris, 1 mM $MgCl_2$, pH 8.0) containing 1 mM dithiothreitol (; DTT), and resuspended in the

electroporation buffer without DTT. The linearized plasmid pTPLR1 (200 ng) was mixed with a 50 μl aliquot (approximately 2×10^7 cells) of the cell suspension, and transferred to a cuvette (0.2-cm electrode gap; Bio-Rad, USA). We performed electroporation (Gene Pulser II; Bio-Rad, USA) under the various ranges of electric pulse (0.8 to 1.2 kV), internal resistance (400 to 800 Ω) and capacitance (25 to 50 μF). The electroporated cells were resuspended in 1 ml of YM medium and transferred to a test tube for incubation. After being shaken for 12 to 16 hours at 23°C, cells were spread on YM agar medium containing 10 μg/ml of cycloheximide and incubated at 23°C for 4 to 5 days.

Figure 4 shows the relationship between the condition of electroporation and the transformation efficiency or cell viability. The transformation efficiency was mainly dependent on the capacitance, preferably of 50 μF rather than 25 μF. In summary, more transformants were produced when an electric pulse of 0.8 kV was delivered and internal resistance of 600 Ω was set with a capacitance of 50 μF, generating pulse lengths of 18 to 20 ms. Under such condition, approximately 30% of cells survived, and transformation efficiencies of 800 to 1000 transformants per μg of DNA could be routinely obtained with pTPLR1 linearized either by *Sma*I or by *Bgl*II-*Kpn*I.

Using the optimized process, we transformed *Phaffia rhodozyma* with various vectors and observed the colony formation on the YM agar medium containing cycloheximide.

Interestingly, there was no transformant with pTPLR2 in any condition, suggesting that L41 gene is expressed only when the transcriptional direction of the integrated L41 gene is the same as that of rDNA.

Without the restriction of pTPLR1 before transformation, no colony was formed. This may result from the fact that rDNA does not have the autonomous replication sequence (; ARS) or its similar function.

A vector carrying cycloheximide-resistant L41 gene but not containing rDNA sequence, was introduced into *Phaffia rhodozyma*. In this case, a few colonies were observed. We suspected that the mutated L41 gene in the vector would replace endogenous L41 gene in the genome, rather than be integrated in directed position.

In addition, we transformed *Phaffia rhodozyma* with a vector in which the promoter of L41 gene was deleted, and observed transformed colonies. The Southern blot analysis of this transformant showed the same hybridization pattern as that of nontransformant control. This indicates that in this case also the transplacement has occurred, rather than be integrated in the directed position.

Example 7: Southern blot analysis of the transformants

To assess the stability of the introduced foreign DNA in *Phaffia rhodozyma* genome according to this invention, we performed Southern blot analysis of genomic DNA, which is prepared from pTPLR1 transformants or nontransformant control (see FIG 5). The genomic DNA was digested with *Sma*I or *Eco*RI enzyme, and the 2.2-kb *Sal*I fragment of pTPL2 was used as a probe in the hybridization. The intensity of colored band was measured by the scanning densitometer (Model GS-700 Imaging Densitometer, Bio-Rad, USA).

Southern blot analysis, in which genomic DNA of transformants was digested with *Sma*I, showed two colored bands at 9.0-kb and 4.1-kb. A signal at 9.0-kb is observed both in a nontransformant control and in the transformants, indicating that this band originated form the endogenous *Phaffia rhodozyma* L41 gene. A much stronger signal at 4.1-kb also was detected in transformants, but not in the control. This was expected from the restriction map of the transforming plasmid (see FIG 6). The size and relative intensity of signal at 4.1-kb suggested that multiple copies (approximately, 7 copies) of the transforming plasmid had been integrated.

In another Southern blot with *Eco*RI digestion, two bands at 5.8-kb and 2.8-kb were found only in

transformants (see FIG 5). The 5.8-kb band originated from a 3.2-kb rDNA fragment and a 2.6-kb L41 gene fragment, and the 2.8-kb band originated from a 1.7-kb rDNA fragment and a 1.1-kb L41 gene fragment. Integration probably occurs as diagrammed in Figure 6.

These results were reproducible in Southern blot with rDNA probe. Most importantly, copy number did not decrease after a prolonged cultivation in YM medium with or without cycloheximide, indicating that the transforming plasmid was integrated into the chromosome and maintained stably.

INDUSTRIAL APPLICABILITY

As shown above, the vectors for transforming *Phaffia rhodozyma* of the present invention comprises rDNA and cycloheximide-resistant L41 gene, which are useful for the stable integration of foreign DNA into host genome and for the convenient selection of transformants, respectively. These vectors are, therefore, applicable to the transformation of yeast cells including *Phaffia rhodozyma*, in combination with the transforming process of this invention, where yeast cells are transformed through the optimized electroporation.

Those skilled in the art will appreciate that the conceptions and specific embodiments disclosed in the foregoing description may be readily utilized as a basis for modifying or designing other embodiments for carrying out the same purposes of the present invention. Those skilled in the art will also appreciate that such equivalent embodiments do not depart from the spirit and scope of the invention as set forth in the appended claims.

What is Claimed is

1. An L41 gene encoding a *Phaffia rhodozyma* ribosomal protein whose amino acid sequence is described by SEQ ID NO: 3.
2. The L41 gene of claim 1, wherein the genomic sequence of the gene is described by SEQ ID NO: 1.
3. The L41 gene of claim 1, wherein the cDNA sequence of the gene is described by SEQ ID NO: 2.
4. The L41 gene of claim 1, wherein the codons representing the amino acid sequence at position 56 is replaced by the codons representing glutamine.
5. A ribosomal DNA of *Phaffia rhodozyma*, which is described by SEQ ID NO: 4.
6. A vector for transforming *Phaffia rhodozyma*, comprising a cycloheximide-resistant gene and a portion of *Phaffia rhodozyma* ribosomal DNA.
7. The vector of claim 6, wherein the cycloheximide-resistant gene is the L41 gene of claim 4.
8. The vector of claim 6, wherein the *Phaffia rhodozyma* ribosomal DNA is the ribosomal DNA of claim 5.
9. The vector of claim 6, wherein the vector is pTPLR1 represented by figure 3.
10. A process of transforming yeast with the vector of claim 6.
11. The process of claim 10, the yeast is *Phaffia*

rhodozyma.

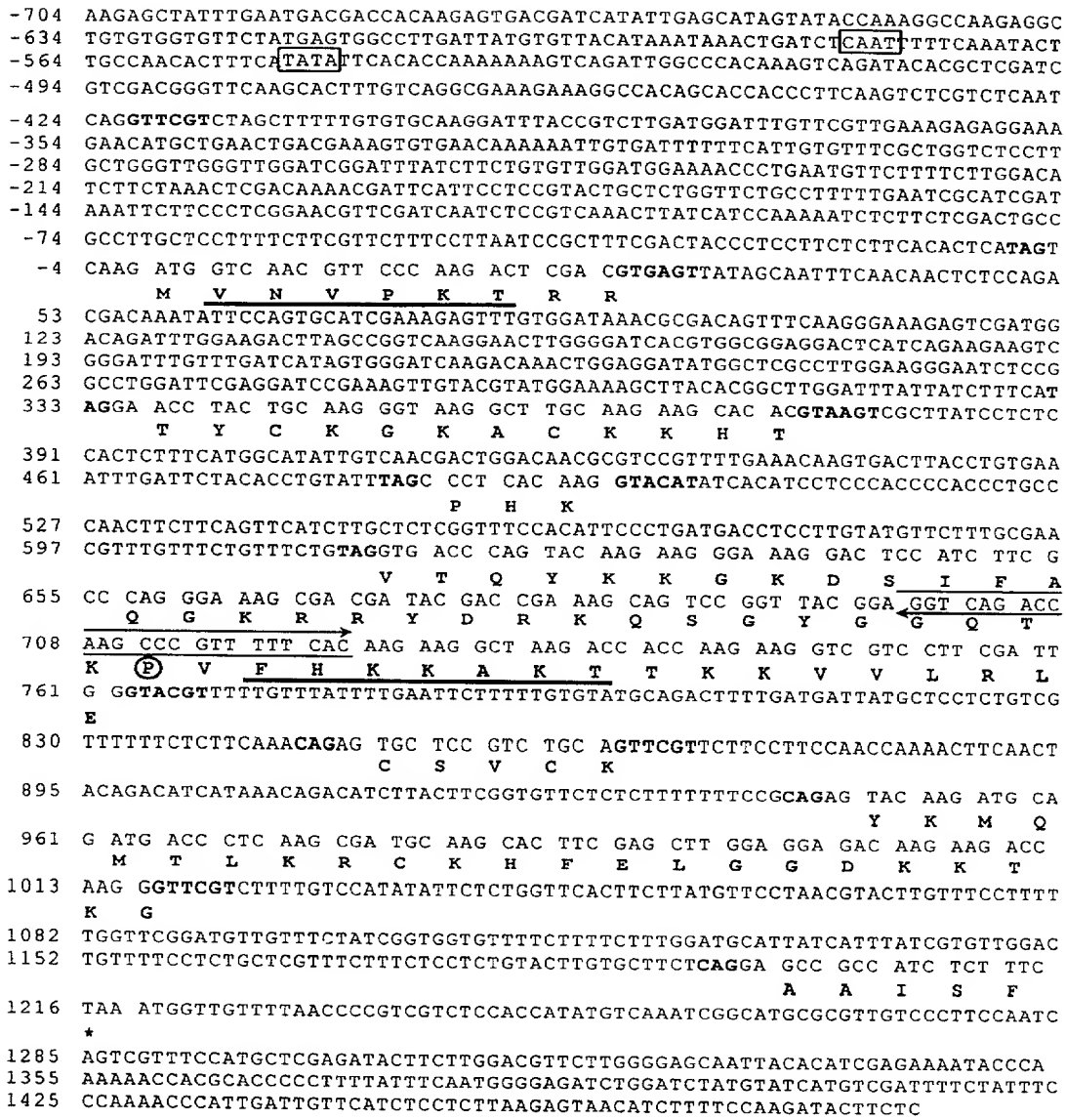
12. The process of claim 10, wherein the vector of claim 6 is cleaved into a linear form.

13. The process of claim 10, wherein the transformation is performed by electroporation under an electric pulse of 0.8~1.2 kV, an internal resistance of 400~800 Ω, and a capacitance of 25~50 μF.

ABSTRACT OF THE DISCLOSURE

The present invention relates to a transforming vector and a process of transformation thereby, more specifically to a trasnsforming vector comprising a cycloheximide-resistant gene and a ribosomal DNA. The transforming vector and the transforming process thereby is applicable to the efficient and stable integration of desired DNA into yeast genome, thus providing useful tools for the production of a natural pigment, astaxanthin.

FIG. 1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-74 GCCTTGCTCCTTTTCTTCGTTCTTTCCTTAATCCGCTTTCGACTACCCTCCTTCTCTTCACACTCATAGT
-4 CAAG ATG GTC AAC GTT CCC AAG ACT CGA CGTGAGTTATAGCAATTTCAACAACTCTCCAGA
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193 GGGATTTGTTTGATCATAGTGGGATCAAGACAAACTGGAGGATATGGCTCGCCTTGGAAGGGAATCTCCG
263 GCCTGGATTCGAGGATCCGAAAGTTGTACGTATGGAAAAGCTTACACGGCTTGGATTTATTATCTTTCAT
333 AGGA ACC TAC TGC AAG GGT AAG GCT TGC AAG AAG CAC ACGTAAGTCGCTTATCCTCTC
T Y C K G K A C K K H T
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P H K
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V T Q Y K K G K D S I F A
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C S V C K
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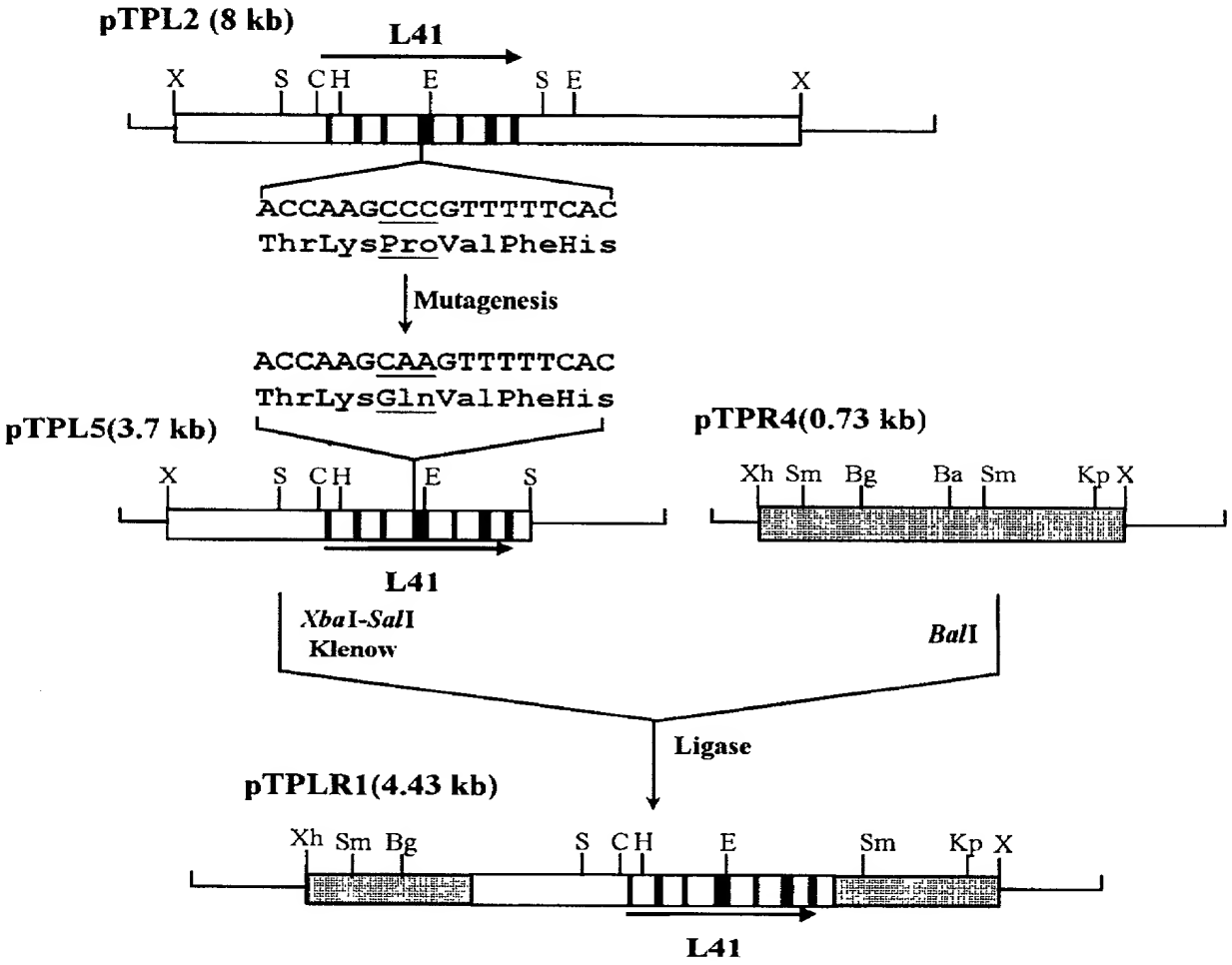
FIG. 2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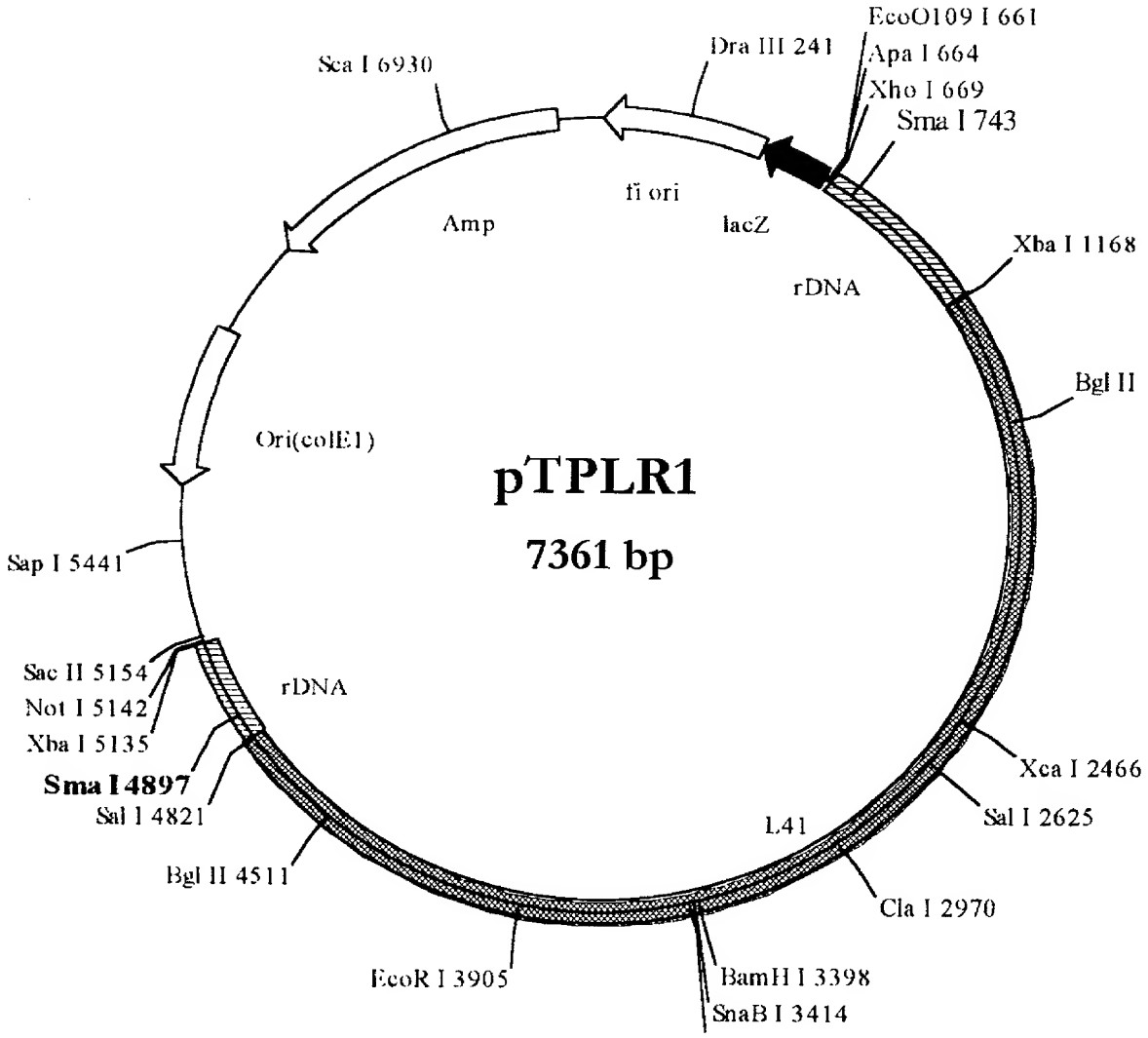
L41

FIG. 3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lacZ
rDNA
Xba I 1168
Bgl II
Ori(colE1)
Sap I 5441
Sac II 5154
Not I 5142
Xba I 5135
Sma I 4897
Sal I 4821
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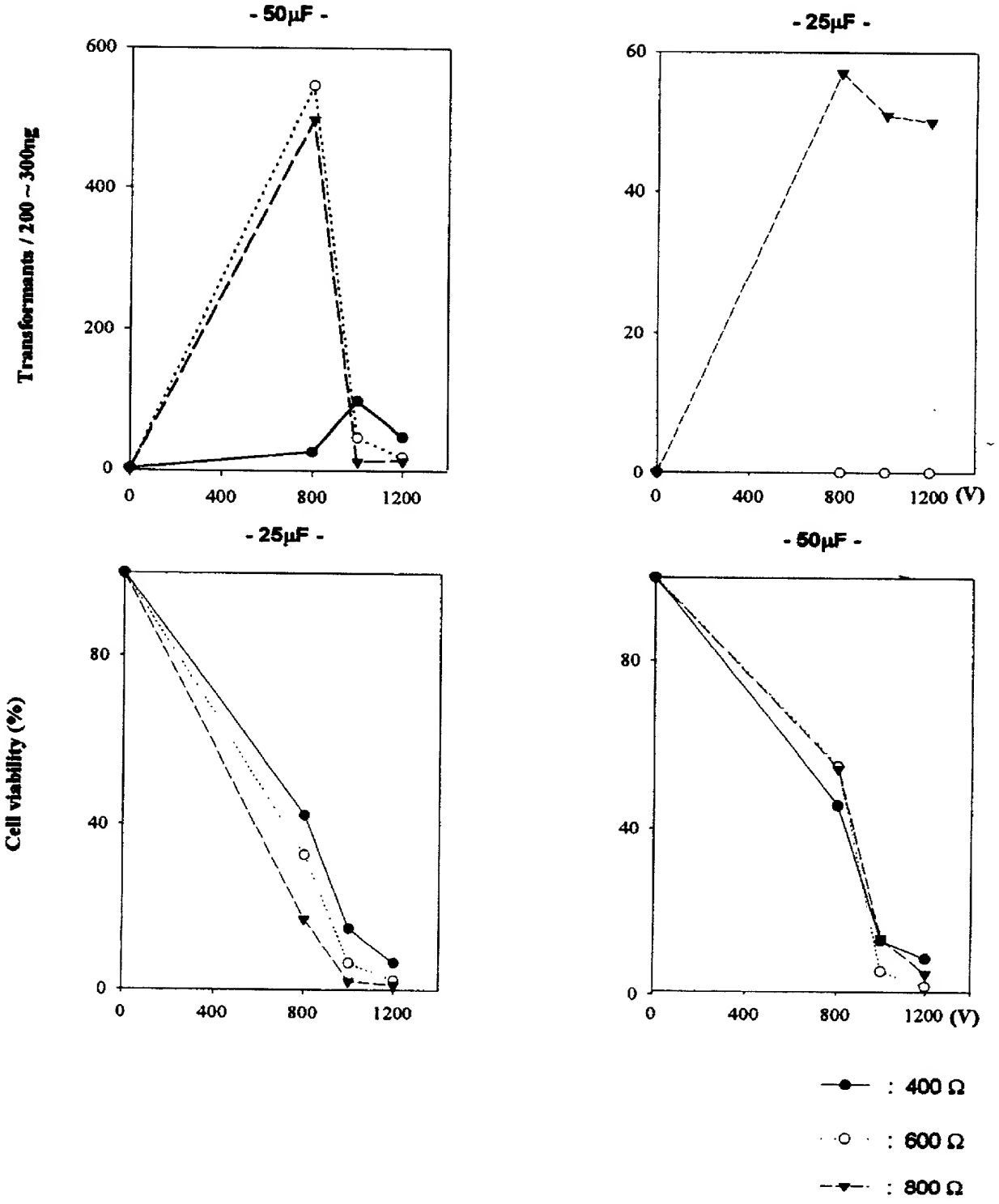
FIG. 4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Transformants / 200 ~ 300ng
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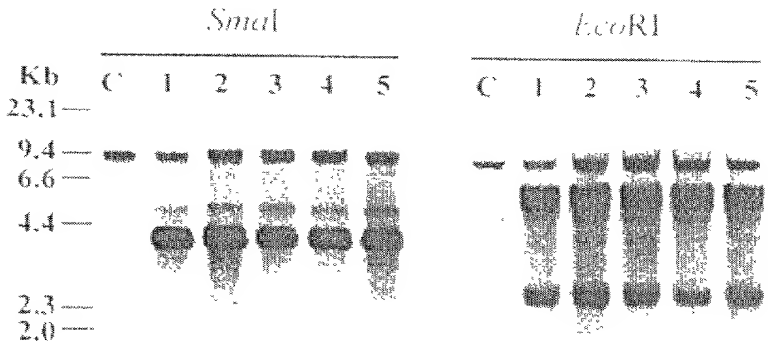
FIG. 5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EcoRI
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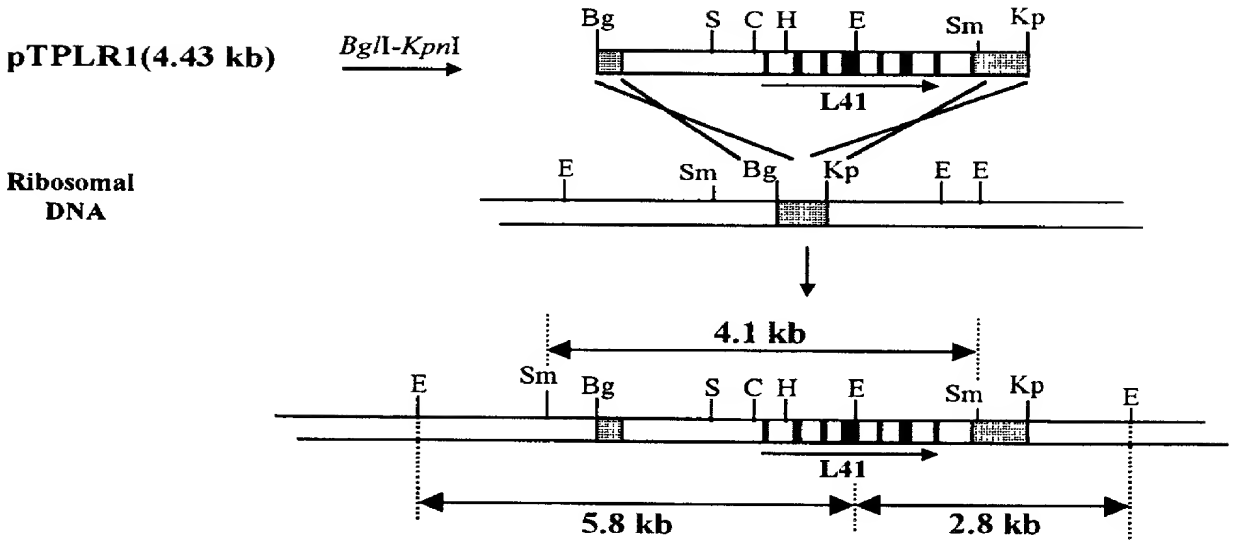
FIG. 6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L41
5.8 kb
2.8 kb

GATES & COOPER LLP

United States Patent Application

COMBINED DECLARATION AND POWER OF ATTORNEY

As a below named inventor I hereby declare that: my residence, post office address and citizenship are as stated below next to my name; that

I verily believe I am the original, first and sole inventor (if only one name is listed below) or a joint inventor (if plural inventors are named below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

VECTOR FOR THE TRANSFORMATION OF PHAFFIA RHODOZYMA AND PROCESS OF TRANSFORMATION THEREBY

TITLE

The specification of which was filed on May 9, 1999 (INTERNATIONAL FILING DATE) as PCT International Application Number PCT/KR99/00265 (INTERNATIONAL APPLICATION NUMBER), which I have reviewed and for which I solicit a United States patent.

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the patentability of this application in accordance with Title 37, Code of Federal Regulations, § 1.56 (attached hereto).

I hereby claim foreign priority benefits under Title 35, United States Code, § 119(a)-(d) or 365(b) of any foreign application(s) for patent or inventor's certificate or 365(a) of any PCT international application which designated at least one country other than the United States of America, listed below and have also identified below any foreign application for patent or inventor's certificate or any PCT application having a filing date before that of the application on the basis of which priority is claimed:

a. ☐ no such applications have been filed.
b. ☒ such applications have been filed as follows:

FOREIGN APPLICATION(S), IF ANY, CLAIMING PRIORITY UNDER 35 USC § 119			
COUNTRY	APPLICATION NUMBER	DATE OF FILING (day, month, year)	DATE OF ISSUE (day, month, year)
KR	1998-46547	October 31, 1998	
OTHER FOREIGN APPLICATION(S), IF ANY, FILED BEFORE THE PRIORITY APPLICATION(S)			
COUNTRY	APPLICATION NUMBER	DATE OF FILING (day, month, year)	DATE OF ISSUE (day, month, year)

I hereby claim the benefit under Title 35, United States Code, § 120 of any United States application(s), or 365(c) of any PCT international application(s) designating the United States of America, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT international application in the manner provided by the first paragraph of Title 35, United States Code, § 112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, § 1.56(a) which occurred between the filing date of the prior application and the national or PCT international filing date of this application.

U.S. PARENT APPLICATION OR PCT PARENT NUMBER	DATE OF FILING (day, month, year)	STATUS (patented, pending, abandoned)

I hereby claim the benefit under Title 35, United States Code § 119(c) of any United States provisional application(s) listed below:

U.S. PROVISIONAL APPLICATION NUMBER	DATE OF FILING (Day, Month, Year)

I hereby appoint the following attorneys to prosecute this application and to transact all business in the Patent and Trademark Office connected herewith:

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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

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Signature of Inventor(3): Shon, jinghoon				Date: Appl. 10. 2001
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Signature of Inventor(4): Park, Soo Dong				Date: April, 10, 2001
(5)	Full Name Of Inventor	Family Name LEE	First Given Name YOON HYOUNG	Second Given Name
	Residence & Citizenship	City Kyoungki-do	State or Foreign Country Republic of Korea	Country of Citizenship Republic of Korea
	Post Office Address	Post Office Address #933-1503 Myohyang Apt., Sanbon-dong, Kunpo-si	City Kyoungki-do	State & Zip Code/Country 435-040 / KR
Signature of Inventor(5): Lee Yoon Hyoung				Date: April 10, 2001

(6)	Full Name Of Inventor	Family Name LEE	First Given Name SEUNG JAE	Second Given Name
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Signature of Inventor(6): Lee Seung Jae				Date: April. 10. 2001
(7)	Full Name Of Inventor	Family Name JANG	First Given Name JAE KWEON	Second Given Name
	Residence & Citizenship	City Seoul	State or Foreign Country Republic of Korea	Country of Citizenship Republic of Korea
	Post Office Address	Post Office Address #102 Jaewon Villa, 229-8 Seokchon-dong, Songpa-ku	City Seoul	State & Zip Code/Country 138-190 / KR
Signature of Inventor(7): JANG Jae-Kweon				Date: April 10, 2001
(8)	Full Name Of Inventor	Family Name CHOI	First Given Name SEOK KEUN	Second Given Name
	Residence & Citizenship	City Seoul	State or Foreign Country Republic of Korea	Country of Citizenship Republic of Korea
	Post Office Address	Post Office Address #503 Jinro Apt., 10 Myunmonk-dong, Chungrang-ku	Seoul	State & Zip Code/Country 131-208 / KR
Signature of Inventor(8): Choi Seok-keun				Date: April 10, 2001
(9)	Full Name Of Inventor	Family Name SON	First Given Name YOUNG ROK	Second Given Name
	Residence & Citizenship	City Seoul	State or Foreign Country Republic of Korea	Country of Citizenship Republic of Korea
	Post Office Address	Post Office Address #919 Nasanmisi 860 Officetel, 13-3 Kaepo-dong, Kangnam-ku	City Seoul	State & Zip Code/Country 135-240 / KR
Signature of Inventor(9): Son Young Rok				Date: April 10, 2001

§ 1.56 Duty to disclose information material to patentability.

(a) A patent by its very nature is affected with a public interest. The public interest is best served, and the most effective patent examination occurs when, at the time an application is being examined, the Office is aware of and evaluates the teachings of all information material to patentability. Each individual associated with the filing and prosecution of a patent application has a duty of candor and good faith in dealing with the Office, which includes a duty to disclose to the Office all information known to that individual to be material to patentability as defined in this section. The duty to disclose information exists with respect to each pending claim until the claim is canceled or withdrawn from consideration, or the application becomes abandoned. Information material to the patentability of a claim that is canceled or withdrawn from consideration need not be submitted if the information is not material to the patentability of any claim remaining under consideration in the application. There is no duty to submit information which is not material to the patentability of any existing claim. The duty to disclose all information known to be material to patentability is deemed to be satisfied if all information known to be material to patentability of any claim issued in a patent was cited by the Office or submitted to the Office in the manner prescribed by §§ 1.97(b)-(d) and 1.98. However, no patent will be granted on an application in connection with which fraud on the Office was practiced or attempted or the duty of disclosure was violated through bad faith or intentional misconduct. The Office encourages applicants to carefully examine:

(1) prior art cited in search reports of a foreign patent office in a counterpart application, and

(2) the closest information over which individuals associated with the filing or prosecution of a patent application believe any pending claim patentably defines, to make sure that any material information contained therein is disclosed to the Office.

(b) Under this section, information is material to patentability when it is not cumulative to information already of record or being made of record in the application, and

(1) it establishes, by itself or in combination with other information, a prima facie case of unpatentability of a claim; or

(2) it refutes, or is inconsistent with, a position the applicant takes in:

(i) opposing an argument of unpatentability relied on by the Office, or

(ii) asserting an argument of patentability.

A prima facie case of unpatentability is established when the information compels a conclusion that a claim is unpatentable under the preponderance of evidence, burden-of-proof standard, giving each term in the claim its broadest reasonable construction consistent with the specification, and before any consideration is given to evidence which may be submitted in an attempt to establish a contrary conclusion of patentability.

(c) Individuals associated with the filing or prosecution of a patent application within the meaning of this section are:

(1) each inventor named in the application;

(2) each attorney or agent who prepares or prosecutes the application; and

(3) every other person who is substantively involved in the preparation or prosecution of the application and who is associated with the inventor, with the assignee or with anyone to whom there is an obligation to assign the application.

(d) Individuals other than the attorney, agent or inventor may comply with this section by disclosing information to the attorney, agent, or inventor.

SEQUENCE LISTING

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Haitai Confectionery Co., Ltd.

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<130> 9fpo-05-02

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<151> 1998-10-31

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Nigel

PCT

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PATENT APPLICATION: US/09/830,691

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  5       Rhee, Sang-Ki
  6       Sohn, Jung-Hoon
  7       Park, Soo-Dong
  8       Lee, Yoon-Hyoung
  9       Lee, Seung-Jae
 10       Jang, Jae-Kweon
 11       Choi, Seok-Keun
 12       Son, Young-Rok
 14 <120> TITLE OF INVENTION: VECTOR FOR THE TRANSFORMATION OF PHAFFIA
 15       RHODOZYMA AND PROCESS OF TRANSFORMATION THEREBY
 18 <130> FILE REFERENCE: 118.12-US-WO
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C--> 21 <141> CURRENT FILING DATE: 2001-04-26
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 24 <151> PRIOR FILING DATE: 1998-10-31
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73 Arg Thr Tyr Cys Lys Gly Lys Ala Cys Lys Lys His Thr Pro His Lys
74      10                  15                  20
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81 Arg Arg Tyr Asp Arg Lys Gln Ser Gly Tyr Gly Gly Gln Thr Lys Pro
82                  45                  50                  55
84 gtt ttc cac aag aag gct aag acc acc aag aag gtc gtc ctt cga ttg      245
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86              60                  65                  70
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89 Glu Cys Ser Val Cys Lys Tyr Lys Met Gln Met Thr Leu Lys Arg Cys
90          75                  80                  85
92 aag cac ttc gag ctt gga gga gac aag aag acc aag gga gcc gcc atc      341
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112         35                  40                  45
113 Gly Tyr Gly Gly Gln Thr Lys Pro Val Phe His Lys Lys Ala Lys Thr
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115 Thr Lys Lys Val Val Leu Arg Leu Glu Cys Ser Val Cys Lys Tyr Lys
116 65                  70                  75                  80
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PATENT APPLICATION: US/09/830,691 TIME: 10:56:06

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VERIFICATION SUMMARY
PATENT APPLICATION: US/09/830,691

DATE: 05/18/2001
TIME: 10:56:07

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Choi, Seok-Keun
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<130> 118.12-US-WO

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<213> Phaffia rhodozyma

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atggtcaacg ttcccaagac tcgacgtgag ttatagcaat ttcaacaact ctccagacga 60
caaatattcc agtgcatcga aagagtttgt ggataaacgc gacagtttca agggaaagag 120
tcgatggaca gatttggaag acttagccgg tcaaggaact tggggatcac gtggcggagg 180
actcatcaga agaagtcggg atttgtttga tcatagtggg atcaagacaa actggaggat 240
atggctcgcc ttggaaggga atctccggcc tggattcgag gatccgaaag ttgtacgtat 300
ggaaaagctt acacggcttg gatttattat ctttcatagg aacctactgc aagggtaagg 360
cttgcaagaa gcacacgtaa gtcgcttatc ctctccactc tttcatggca tattgtcaac 420
gactggacaa cgcgtccgtt ttgaaacaag tgacttacct gtgaaatttg attctacacc 480
tgtatttagc cctcacaagg tacatatcac atcctcccac cccaccctgc ccaacttctt 540
cagttcatct tgctctcggt ttccacattc cctgatgacc tccttgtatg ttctttgcga 600
acgtttgttt ctgtttctgt aggtgaccca gtacaagaag ggaaaggact ccatcttcgc 660
ccagggaaag cgacgatacg accgaaagca gtccggttac ggaggtcaga ccaagcccgt 720
tttccacaag aaggctaaga ccaccaagaa ggtcgtcctt cgattggcgg tatttttgtt 780
tattttgaat tctttttgtg tatgcagact tttgatgatt atgctcctct gtcgtttttt 840
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cagacatcat aaacagacat cttacttcgg tgttctctct ttttttccgc agagtacaag 960
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ccttttggt tcggatgttg tttctatcgg tggtgttttc ttttctttgg atgcattatc 1140
atttatcgtg ttggactgtt ttcctctgct cgtttctttc tcctctgtac ttgtgcttct 1200
caggagccgc catctctttc taa 1223

<210> 2
<211> 350
<212> DNA
<213> Phaffia rhodozyma

<220>
<221> CDS
<222> (30)...(347)

<400> 2
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ccccttcaagt ctcgtctcaa tcagtcaag atg gtc aac gtt ccc aag act cga        53
                                   Met Val Asn Val Pro Lys Thr Arg
                                    1               5

cga acc tac tgc aag ggt aag gct tgc aag aag cac acc cct cac aag       101
Arg Thr Tyr Cys Lys Gly Lys Ala Cys Lys Lys His Thr Pro His Lys
     10                  15                  20

gtg acc cag tac aag aag gga aag gac tcc atc ttc gcc cag gga aag       149
Val Thr Gln Tyr Lys Lys Gly Lys Asp Ser Ile Phe Ala Gln Gly Lys
 25                  30                  35                  40

cga cga tac gac cga aag cag tcc ggt tac gga ggt cag acc aag ccc       197
Arg Arg Tyr Asp Arg Lys Gln Ser Gly Tyr Gly Gly Gln Thr Lys Pro
                 45                  50                  55

gtt ttc cac aag aag gct aag acc acc aag aag gtc gtc ctt cga ttg       245
Val Phe His Lys Lys Ala Lys Thr Thr Lys Lys Val Val Leu Arg Leu
             60                  65                  70

gag tgc tcc gtc tgc aag tac aag atg cag atg acc ctc aag cga tgc       293
Glu Cys Ser Val Cys Lys Tyr Lys Met Gln Met Thr Leu Lys Arg Cys
         75                  80                  85

aag cac ttc gag ctt gga gga gac aag aag acc aag gga gcc gcc atc       341
Lys His Phe Glu Leu Gly Gly Asp Lys Lys Thr Lys Gly Ala Ala Ile
     90                  95                  100

tct ttc taa                                                          350
Ser Phe
105
```

<210> 3
<211> 106
<212> PRT
<213> Phaffia rhodozyma

<400> 3
```
Met Val Asn Val Pro Lys Thr Arg Arg Thr Tyr Cys Lys Gly Lys Ala
 1               5                  10                  15
Cys Lys Lys His Thr Pro His Lys Val Thr Gln Tyr Lys Lys Gly Lys
            20                  25                  30
Asp Ser Ile Phe Ala Gln Gly Lys Arg Arg Tyr Asp Arg Lys Gln Ser
        35                  40                  45
Gly Tyr Gly Gly Gln Thr Lys Pro Val Phe His Lys Lys Ala Lys Thr
    50                  55                  60
```

```
Thr Lys Lys Val Val Leu Arg Leu Glu Cys Ser Val Cys Lys Tyr Lys
65                  70                  75                  80
Met Gln Met Thr Leu Lys Arg Cys Lys His Phe Glu Leu Gly Gly Asp
                85                  90                  95
Lys Lys Thr Lys Gly Ala Ala Ile Ser Phe
            100                 105
```

<210> 4
<211> 741
<212> DNA
<213> Phaffia rhodozyma

<220>
<221> misc_feature
<222> (0)...(0)
<223> n=a, t, c, or g

<400> 4

```
ctcgagtgga cggtggcaat ggcattcgtg tcgttggtgc tcactcgcaa cccaagcagt      60
cgcttacccg gggtagcctc cgggtgggcg cgatgatttg tggtgtggat tccttcccta     120
tgggtagaac gacgcgcaac caatcattcg gagaaccgct ccgttgtagc cgaccagtct     180
gattgatcaa catgccagca cgtcctccgg gacggagact ggcggggatc gtacctcatc     240
tggaatcgct ggctcaatgg tagtagtctt cacgatcggc catgagggca gtctaggtgg     300
gttcgcctgc cgaagactgt gtgagtgtgc tganaactaa ttgagtaccg ggggataagg     360
caaggcgtgt ntggttgccg gtggctgtga gcgagtttgc tgcaaagcga ttcaatgcac     420
cccggcttgg ccagcgcgct gcgtcacgaa acacactaaa cggttgacgc cataaagtaa     480
taacacactc aagtttgtgg tcccgggtgg gcctctgtgc ctgcgtggga cccgacggga     540
gaggaaaacg ttctgtggcc ctctcctctg tggatagtta cctggttgat cctgccagta     600
gtcatatgct tgtctcaaag attaagccat gcatgtctaa gtataaacaa attcatactg     660
tgaaactgcg aatggctcat taaatcagtt atagtttatt tgatggtacc ttgctacatg     720
gataactgtg gtaattctag a                                               741
```

<210> 5
<211> 23
<212> DNA
<213> Artificial Sequence

<220>
<223> CYH1, a PCR primer for the cloning of L41 genomic
DNA fragment

<221> misc_feature
<222> (0)...(0)
<223> n=a, t, c, or g

<400> 5

```
cgcgtagtta aygtnccnaa rac                                             23
```

<210> 6
<211> 25
<212> DNA
<213> Artificial Sequence

<220>
<223> CYH3, a PCR primer for the cloning of L41 genomic
DNA fragment

<400> 6
cccgggtytt ggcyttyttr tgraa 25

<210> 7
<211> 24
<212> DNA
<213> Artificial Sequence

<220>
<223> 3' RACE primer

<400> 7
ggtcagacca agcaagtttt tcac 24

<210> 8
<211> 24
<212> DNA
<213> Artificial Sequence

<220>
<223> 5' RACE primer

<400> 8
gtgaaaaact tgcttggtct gacc 24

<210> 9
<211> 24
<212> DNA
<213> Artificial Sequence

<220>
<223> sense primer for the mutagenesis of L41 gene

<400> 9
ggtcagacca agcaagtttt tcac 24

<210> 10
<211> 24
<212> DNA
<213> Artificial Sequence

<220>
<223> antisense primer for the mutagenesis of L41 gene

<400> 10
gtgaaaaact tgcttggtct gacc 24

<210> 11
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> a PCR primer corresponding to 18S rDNA

<400> 11
tcctagtaag cgcaagtcat 20

<210> 12
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> a PCR primer corresponding to 18S rDNA

<400> 12
ttcggccaag gaaagaaact 20

<210> 13
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> a PCR primer corresponding to 28S rDNA

<400> 13
aatcggatta tccggagcta 20

<210> 14
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> a PCR primer corresponding to 28S rDNA

<400> 14
gctataacac atccggagat 20